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C-CAM1 (also named Ceacam1) is a cell adhesion molecule of the immunoglobulin supergene family. We have shown that C-CAM1 plays critical roles in prostate cancer initiation and progression and that loss of C-CAM1 is an early event in the development of prostate cancer. Although tumorigenesis studies in mouse xenograft model have suggested the involvement of C-CAM1 in epithelial cell growth and differentiation, the functional roles of C-CAM1 in normal prostate development, prostate homeostasis, and prostate tumorigenesis remain unclear. We propose to determine the roles of C-CAM1's growth suppressive activity in prostate growth and tumorigenesis by using gene targeting and embryonic stem cell technologies to generate C-CAM1 knockout mice. We have designed a gene targeting strategy that is specific to Ceacam1 gene. In addition, the Ceacam1 gene in the targeted vector was flanked by loxP sites to allow for generating both straight and conditional knockout of Ceacam1 gene. The targeting vector has been constructed and 24 embryonic stem cell clones containing the recombinant gene allele have been established. Three of the embryonic cell clones have been injected into blastocysts for germ line transmission of the targeting construct.

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## (4) INTRODUCTION

C-CAM is a cell adhesion molecule of the immunoglobulin supergene family [1]. We have recently shown that C-CAM plays critical roles in prostate cancer initiation and progression and that loss of C-CAM is an early event in the development of prostate cancer [2]. Although tumorigenesis studies in mouse xenograft model have suggested the involvement of C-CAM in epithelial cell growth and differentiation, the functional roles of C-CAM in normal prostate development, prostate homeostasis, and prostate tumorigenesis remain unclear. Towards the aim of determining the roles of C-CAM's growth suppressive activity in prostate growth and tumorigenesis, we propose to use gene targeting and embryonic stem cell technologies to generate C-CAM knockout mice. Specifically, we plan (1) to determine the roles of C-CAM's growth suppressive function in vivo by generating mice with a targeted deletion of the C-CAM cytoplasmic domain; (2) to determine the roles of C-CAM's growth suppressive function in prostate development and tumorigenesis by generating mice with a prostate-specific knockout of the C-CAM cytoplasmic domain. The proposed work was divided into two Tasks to be carried out in parallel.

**Task 1**. Generate mice with targeted deletion of C-CAM cytoplasmic domain to determine the roles of C-CAM's growth suppressive function in vivo (months 1-30)

**Task 2**. Prostate-specific loss of function of C-CAM gene in prostate (months 7-36)

Genetic manipulation of mouse genes in vivo is a powerful approach for understanding the function of a gene, both during embryonic development and in adult tissues. This method requires full knowledge of the genomic structure of the gene of interest. Unlike humans and rats, which each have one C-CAM1 (renamed as Ceacam1 [3]) gene, two Ceacam1-like genes,

Ceacam1 and Ceacam2, were identified in mice. This poses potential problems in any attempt to manipulate these genes. First, if the two genes have the same function, deletion of one gene may not produce any phenotypic alteration. Second, if there is no significant difference in the genes' sequences, any genetic manipulation may not be specific for a single gene, or it might be difficult to ascertain which gene was altered. It is, therefore, essential to determine the complete sequences and expression profiles of these related Ceacam genes in the 129sv mouse strain before genetic manipulation performed.

In the previous study, we have isolated and sequenced two closely related Ceacam (C-CAM) genes, i.e. Ceacam1 and Ceacam2, from a mouse 129 Sv/Ev library [4]. We have also examined the tissue-specific and embryonic expressions of these mouse Ceacam1 and Ceacam2 genes [4]. Our sequence analysis revealed that the genes encoded nine exons and spanned approx. 16-17 kb (Ceacam1) and 25 kb (Ceacam2). The genes were highly similar (79.6%). The major differences in the protein-coding regions were located in exons 2, 5 and 6. To determine whether functional redundancy exists between Ceacam1 and Ceacam2, we examined their expression in 16 mouse tissues by using semi-quantitative reverse transcription-PCR. As in human and rat, in the mouse Ceacam1 mRNA was highly abundant in the liver, small intestine, prostate and spleen. In contrast, Ceacam2 mRNA was only detected in kidney, testis and, to a lesser extent, spleen. In the mouse embryo, Ceacam1 mRNA was detected at day 8.5, disappeared between days 9.5 and 12.5, and re-appeared at day 19. On the other hand, no Ceacam2 mRNA was detected throughout embryonic development. The different tissue expression patterns and regulation during embryonic development suggest that the CEACAM1 and CEACAM2 proteins, although highly similar, may have different functions both during mouse development and in adulthood.

Results from this study allow us to design a gene targeting strategy that is specific to Ceacam1 gene and also allow us to perform both straight and conditioned knockout of Ceacam1 gene in parallel. In this study, we have designed a gene targeting strategy that is specific to Ceacam1 gene. In addition, the Ceacam1 gene in the targeted vector was flanked by loxP sites to allow for generation of both straight and conditional knockout of Ceacam1 gene. The targeting vector has been constructed and 24 embryonic stem cell clones containing the recombinant gene allele have been established. Three of the embryonic cell clones have been injected into blastocysts for germ line transmission of the targeting construct.

**(5) BODY (Progress report)**

Because Task 1 and Task 2 are being performed in parallel, we describe the progresses in these aspect together.

5. 1. Screening of a BAC library for the isolation of Ceacam genes.

To construct the targeting vector for the knockout of Ceacam1 gene, we screened a 129Sv/Ev mouse genomic library in BAC vector using a probe generated from cDNA coding for full-length Ceacam1. Three positive BAC clones were identified and their structure were determined by restriction mapping. Consistent with our previous study, two closely related genes, i.e. Ceacam1 and Ceacam2, were identified from restriction mapping.

5. 2. Development of gene targeting strategy

In our previous study, we have examined the expression patterns of Ceacam1 and Ceacam2 genes in adult mouse tissues and during embryonic development. Several conclusions can be made from the study. First, the *Ceacam1* and *Ceacam2* genes contained sufficient

sequence differences that targeted gene deletion specific to either gene should be feasible. Second, functional redundancy may not be a problem when only one of these two genes is deleted because although *Ceacam1* and *Ceacam2* were highly homologous, they had different tissue expression patterns. Third, *Ceacam1* is probably more important than *Ceacam2* in the mouse because *Ceacam1's* tissue expression pattern was similar to those of the single Ceacam genes in humans and rats. Conservation of the expression profile among these different species suggests that *Ceacam1's* function may be essential. Fourth, *Ceacam2* plays no role in embryonic development; it is not expressed in the mouse embryo. Thus, it seems that Ceacam1 is functionally more important than Ceacam2 gene.

Knocking out a gene can have no phenotypic effect if related genes have similar functions. We showed that *Ceacam1* and *Ceacam2* were expressed in different tissues in the mouse. *Ceacam1* message was detected in tissues rich in epithelial cells which is consistent with expression of the CEACAM1 homologue in rats and humans [5-7]. In contrast, *Ceacam2* message was abundantly expressed in testis, which does not express *Ceacam1* message. In addition, *Ceacam2* message was undetectable in the mouse embryo, whereas *Ceacam1* message was developmentally regulated. Thus, it appears that the two mouse *Ceacam* genes are not functionally redundant. However, we cannot rule out the possibility that the loss of *Ceacam1* expression may upregulate expression of *Ceacam2*. These studies would have to await targeted gene deletion of these two related genes.

5. 3. Gene targeting strategy

The results of the previous study provide important information for designing gene targeting strategies for functional studies of *Ceacam* genes. Based on the information from the

genomic characterization of Ceacam1 and Ceacam2 gene, we decided to delete the cytoplasmic domain of Ceacam1. This strategy is due to the following reasons: (1) The Ceacam1 gene is functionally more important than Ceacam2 gene; (2) The entire Ceacam1 gene, which is around 20 kb, is too large to delete; (3) The cytoplasmic domain of CEACAM1 is critical for tumor suppressor function. The knockout construct planned for is a conditional knockout construct deleting exon 7~9. This way we will have the option of deleting this region both in ES cells and in the mice. For straight knockout, which is the goal of Task 1, we will transfect the cre recombinase expression plasmid to the ES cells and screen for ES cells that have deletions in exon 7-9 of its Ceacam1 gene before injection the ES cells to blastocysts. For the conditional knockout, which is the goal of Task 2, the ES cells that has targeting vector will be injected into blastocysts and the mice carry the conditional Ceacam1 allele will be generated. The Ceacam1 gene will be deleted in prostate by crossing with transgenic mouse carrying probasin-driven cre recombinase. Therefore, Task 1 and Task 2 are being performed in parallel. The gene knockout strategy is shown in Fig. 1.

5. 4. Targeting vector construction

There are 4 steps in the vector construction strategy. First, the 3.4 kb HindIII-SacI 3'-arm fragment from subclone SacI-1, 3.7 kb XmnI-HindIII middle fragment from subclone HindIII-8 and 4.3 kb XmnI-XmnI 5'-arm fragment from subclone HindIII-8 were cloned into pZero-2 vector, respectively. These were necessary for the easy vector construction and later DNA sequencing confirmation. Second, the 3.4 kb HindIII-SacI 3'-arm fragment was subcloned into 38LoxPNeo vector and the 3'-arm vector with correct orientation was obtained. Third, the 3.7 kb XmnI-HindIII middle fragment was cloned into BamHI site of the 3'-arm vector and the mid-3'-vector with right orientation was obtained. Finally, the 4.3 kb XmnI-XmnI 5'-arm fragment was

cloned into XhoI site of the mid-3'-vector for the construction of the final targeting vector. The targeting vector was verified by restriction mapping (Fig. 2).

5. 5. Transfection and generation of embryonic stem cells containing targeting vector

The targeting vector was transfected into embryonic stem cells by electroporation. A total of 270 ES cell clones were obtained from two electroporations. Genomic DNA was extracted from these ES cell clones. Half of the DNA from each sample was digested with SphI and processed for Southern blot analysis. The 5' probe used is a 0.7kb ApaI/SpeI fragment from subclone BamHI-A. Using the 5' probe, a ~9.2 kb band corresponding to the endogenous allele of Ceacam-1 and a ~10kb band corresponding to Ceacam-2 could be visualized in most samples. Many clones (a total of 24) contained an additional hybridization signal at the size predicted for the homologous recombinant allele, 8.1 kb (Fig. 3). The clones identified as potential positives using the 5' probe were digested with Xho I and hybridized with the 3' probe, which is 1.2kb Sac I/Xho I fragment from subclone Apa I-4. A ~15.3 kb band corresponding to the endogenous allele of Ceacam-1 and a ~17.1 kb band corresponding to recombinant allele were detected in 16 clones (Fig. 4). These 16 clones were also positive with Neo probe, which gave a 17.1 kb band in Southern blot analysis. Taken together, a total of 16 ES cell clones containing the Ceacam1 conditional allele were generated.

5. 6. Injection into mouse blastocysts

Three ES cell clones were injected into mouse blastocysts using procedures as described in Chang et al. [8]. Briefly, chimeric mice are generated by microinjection of 8-20 ES cells into the blstocoel of embryonic d 3.5 C57Bl/6J mouse embryos. The embryos are transferred to the uteri of postcoitum d 2.5 pseudopregnant ICR mice, and the embryos are allowed to gestate. Ten mice were born from these mice and will be genotyped.

## (6) KEY RESEARCH ACCOMPLISHMENTS

- Screen a mouse 129Sv/Ev genomic library in BAC vector and isolate several BAC clones.
- Map the BAC clones.
- Design knockout strategy that allows both Task1 and Task2 being carried out in parallel.
- Construct the targeting vector.
- Transfect the targeting vector into embryonic stem cells and selected 24 positive ES cell clones.
- Inject three ES cell clones into blastocysts.

## (7) REPORTABLE OUTCOMES

Han, E., Phan, D., Lo, P., Poy, M. N., Behringer, R., Najjar, S., and Lin, S.-H.: Difference in tissue-specific and embryonic expression of mouse Ceacam1 and Ceacam2 genes. Biochem. J. (2001) 355, 417-423.

**(8) CONCLUSION** We propose to determine the roles of CEACAM's growth suppressive activity in prostate growth and tumorigenesis by using gene targeting and embryonic stem cell technologies to generate CEACAM knockout mice. We have designed and constructed a gene targeting vector that is specific to Ceacam1 gene. This gene targeting strategy will allow us to perform both straight and conditioned knockout of Ceacam1 gene in parallel. Embryonic stem cells containing the knockout construct have been generated and injected into blastocysts. The mice carrying Ceacam1 conditional knock out allele are being generated.

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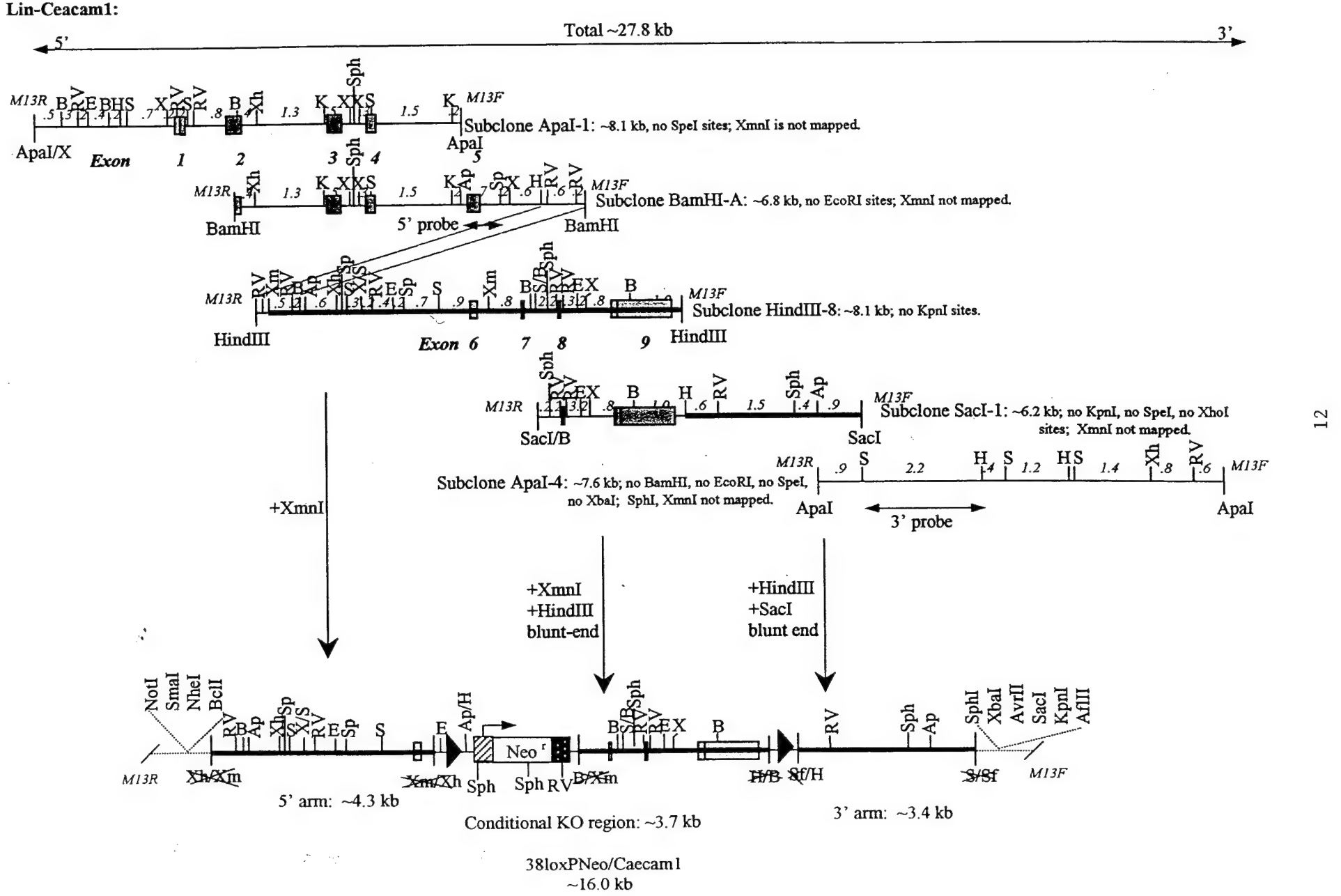
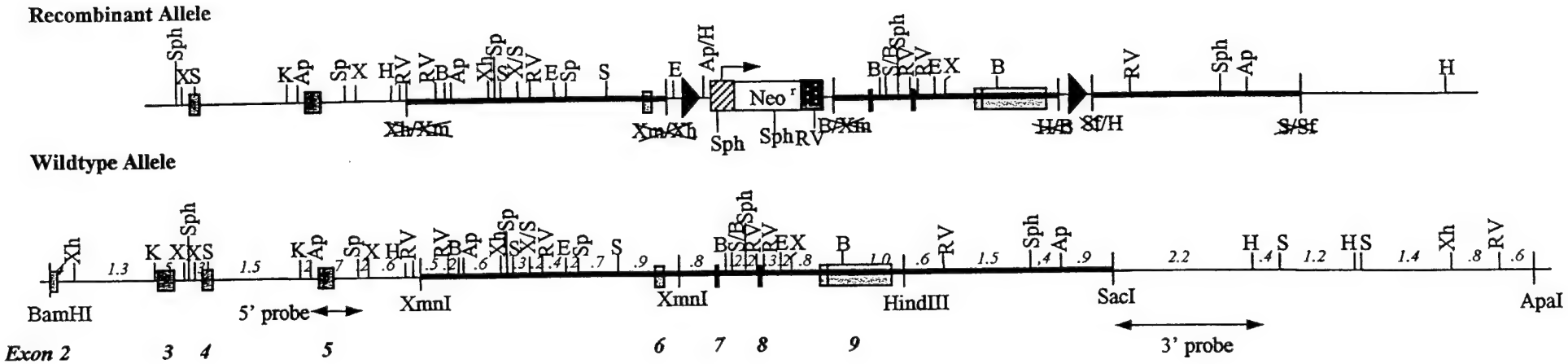
**Fig. 1. Ceacam1 gene knockout strategy.**





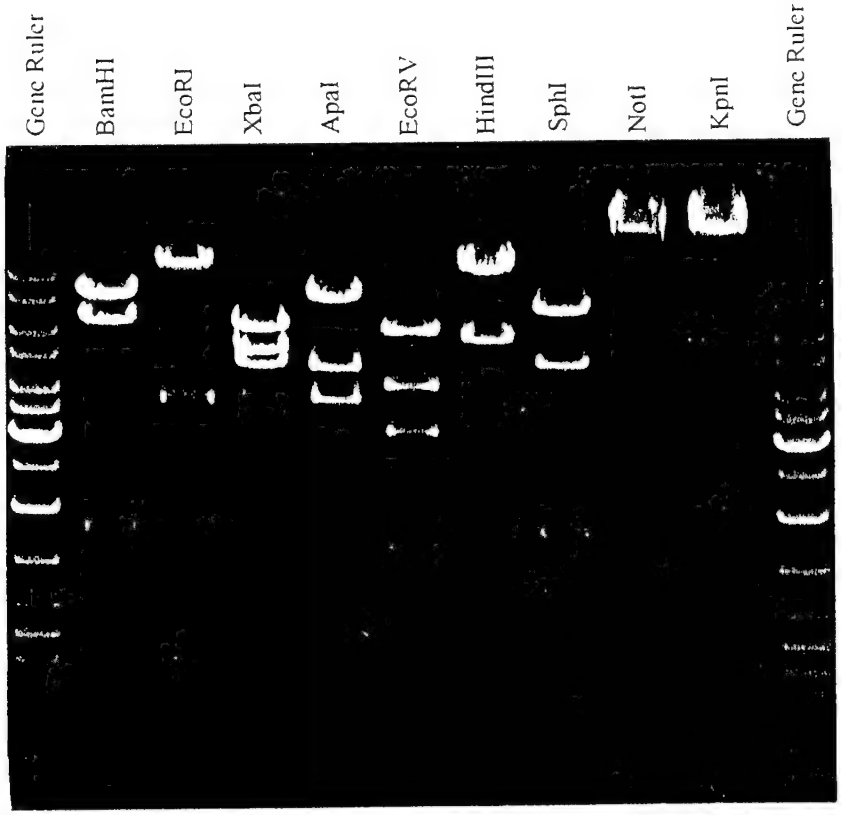

**Screening Strategy**:

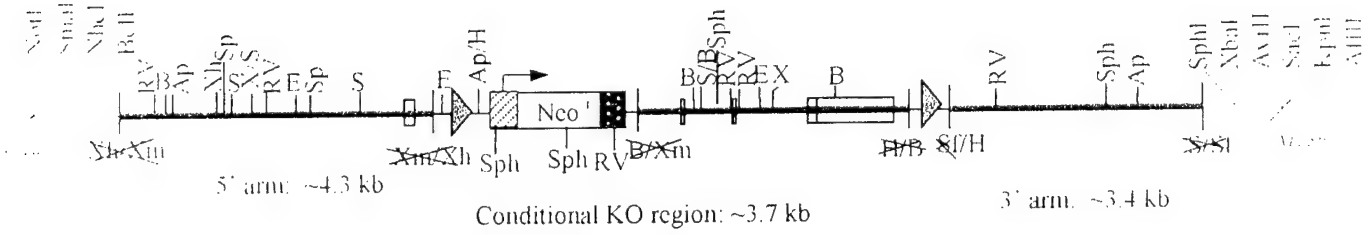
| | | |
|---|---|---|
| 5' probe: | SphI digestion for genomic DNA | |
| | wildtype allele: | 9.2 kb |
| | recombinant allele: | 8.2 kb |
| 3' probe: | XhoI digestion for genomic DNA | |
| | wildtype allele: | 15.5 kb |
| | recombinant allele: | 17.4 kb |

Note: Ap=ApaI; B=BamHI; E=EcoRI; H=HindIII; K=KpnI; RV=EcoRV; S=SacI; Sf=SfiI; Sp=SpeI; Sph=SphI; X=XbaI; Xh=XhoI; Xm=XmnI.

1 kb ▬ LoxP site ▶

**Fig. 2. Restriction mapping of targeting vector.**




Abbreviation: Ap=ApaI; B=BamHI; E=EcoRI; H=HindIII; K=KpnI; RV=EcoRV; S=SacI; Sf=SfiI; Sp=SpeI; Sph=SphI; X=XbaI; Xh=XhoI; Xm=XmnI.

1 kb

NotI or KpnI can be used to linearize the final targeting vector.

**Fig. 3. Screening of ES cells with homologous recombinant allele.** The genomic DNA was digested with SphI and hybridized with 5' probe. Clone 137 is a wild-type ES cells while clone 138 contains Ceacam1 recombinant allele.

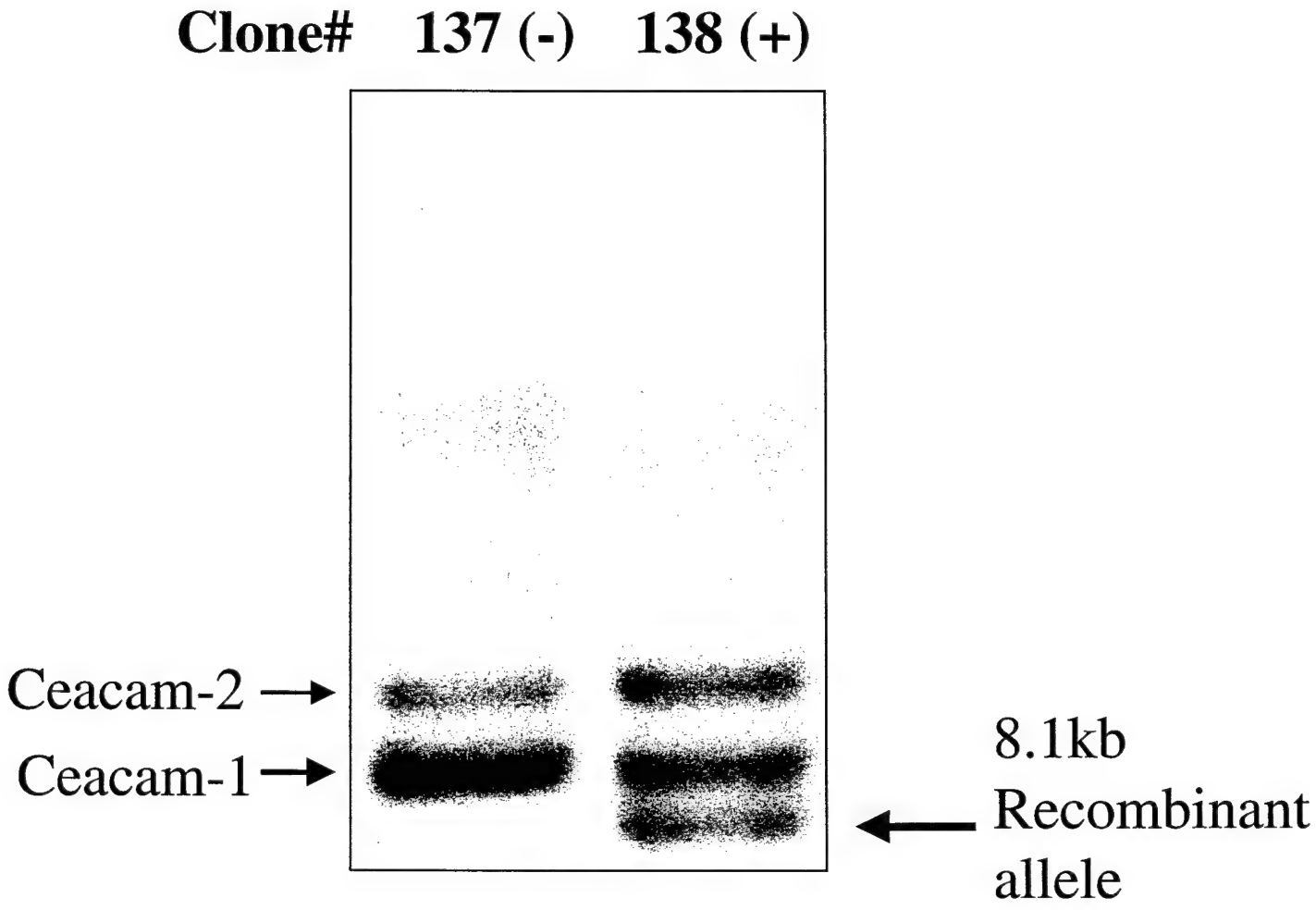
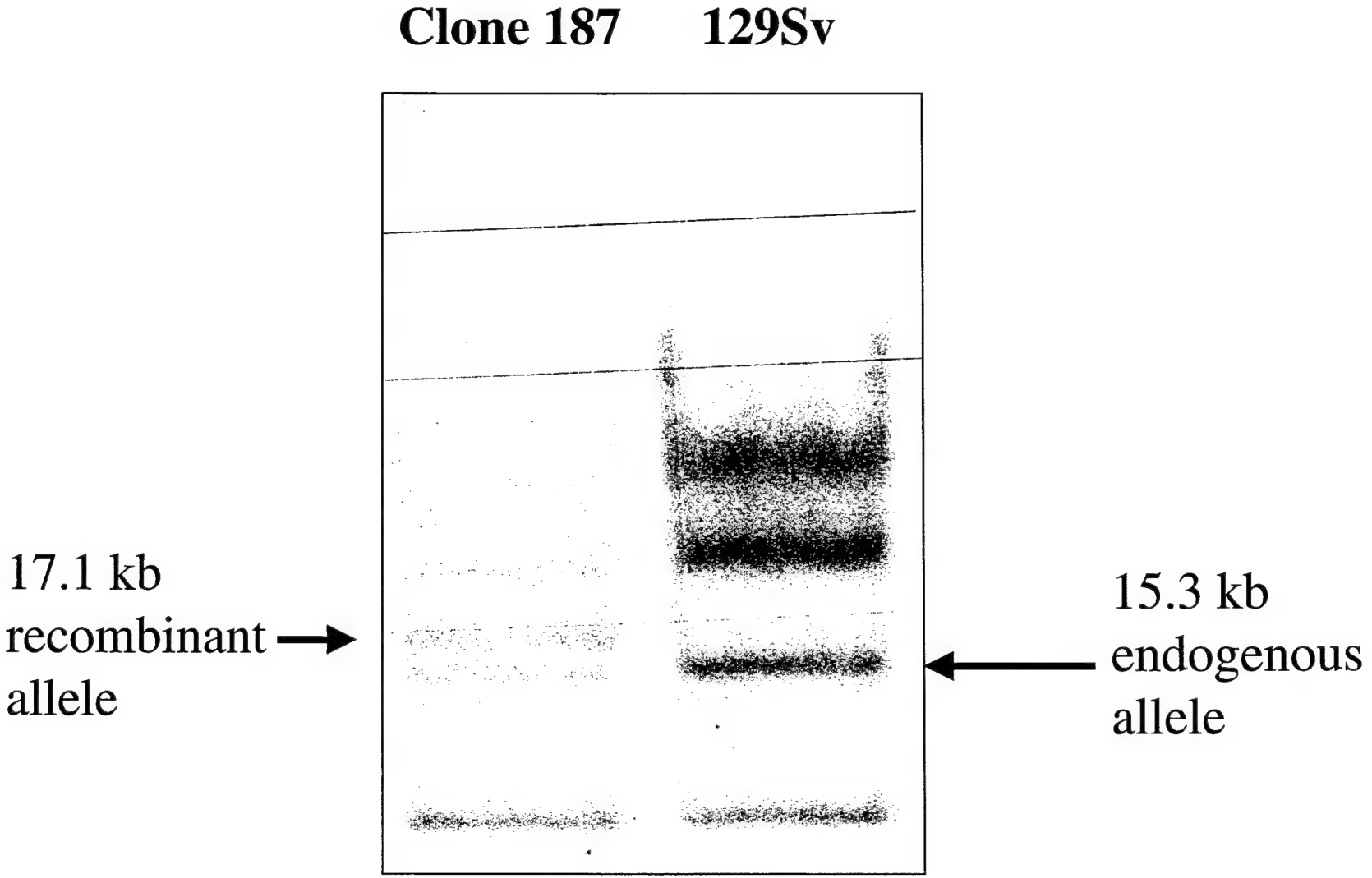


**Fig. 4. Screening of ES cells with homologous recombinant allele.**
The genomic DNA was digested with XhoI and hybridized with 3' probe.



# Differences in tissue-specific and embryonic expression of mouse *Ceacam1* and *Ceacam2* genes

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The intercellular adhesion molecule CEACAM1, also known as C-CAM1 (where CAM is cell-adhesion molecule), can function as a tumour suppressor in several carcinomas, including those of the prostate, breast, bladder and colon. This suggests that CEACAM1 may play an important role in the regulation of normal cell growth and differentiation. However, there is no direct evidence to support this putative function of CEACAM1. To elucidate its physiological function by targeted gene deletion, we isolated the *Ceacam* genes from a mouse 129 Sv/Ev library. Although there is only one *Ceacam1* gene in humans and one in rats, two homologous genes (*Ceacam1* and *Ceacam2*) have been identified in the mouse. Our sequence analysis revealed that the genes encoded nine exons and spanned approx. 16–17 kb (*Ceacam1*) and 25 kb (*Ceacam2*). The genes were highly similar (79.6%). The major differences in the protein-coding regions were located in exons 2, 5 and 6 (76.9%, 87.0% and 78.5% similarity respectively). In addition, introns 2, 5 and 7 were also significantly different, being 29.7%, 59.8% and 64.5% similar respectively. While most of these differences were due to nucleotide substitutions, two insertions of 418 and 5849 bp occurred in intron 2 of *Ceacam2*, and another two insertions of 1384 and 197 bp occurred in introns 5 and 7 respectively. To determine whether functional redundancy exists between *Ceacam1* and *Ceacam2*, we examined their expression in 16 mouse tissues by using semi-quantitative reverse transcription–PCR. As in human and rat, in the mouse *Ceacam1* mRNA was highly abundant in the liver, small intestine, prostate and spleen. In contrast, *Ceacam2* mRNA was only detected in kidney, testis and, to a lesser extent, spleen. Reverse transcription–PCR using testis RNA indicated that *Ceacam2* in the testis is an alternatively spliced form containing only exons 1, 2, 5, 6, 8 and 9. In the mouse embryo, *Ceacam1* mRNA was detected at day 8.5, disappeared between days 9.5 and 12.5, and re-appeared at day 19. On the other hand, no *Ceacam2* mRNA was detected throughout embryonic development. The different tissue expression patterns and regulation during embryonic development suggest that the CEACAM1 and CEACAM2 proteins, although highly similar, may have different functions both during mouse development and in adulthood.

Key words: C-CAM, cell-adhesion molecule, gene expression, tumour suppressor.

## INTRODUCTION

The intercellular adhesion molecule CEACAM1, also known as C-CAM1 [1] (where CAM is cell-adhesion molecule), is a member of the Ig gene family [2]. In addition to its cell-adhesion function, CEACAM1 is also a tumour suppressor in prostate [3], breast [4], bladder [5] and colon [6] carcinomas. Consistent with its role as a tumour suppressor, loss of CEACAM1 expression was observed in hepatomas [7], colon carcinomas [8–10] and endometrial [11] and prostate [12,13] cancers. This suggests that CEACAM1 may regulate cell growth and differentiation.

To elucidate the physiological functions of CEACAM1, we plan to generate mice with targeted *Ceacam* deletions. Genetic manipulation of mouse genes *in vivo* is a powerful approach for understanding the function of a gene, both during embryonic development and in adult tissues. This method requires full knowledge of the genomic structure of the gene of interest. Unlike humans and rats, which each have one *Ceacam1* gene [14–16], two *Ceacam1*-like genes, *Ceacam1* and *Ceacam2*, were identified in BALB/c mice [17]. This poses potential problems in any attempt to manipulate these genes. First, if the two genes have the same function, deletion of one gene may not produce any phenotypic alteration. Secondly, if there is no significant difference in the sequences of the genes, any genetic manipulation may not be specific for a single gene, or it might be difficult to ascertain which gene was altered. It is, therefore, essential to determine the complete sequences and expression profiles of these related *Ceacam* genes in the 129 Sv mouse strain before genetic manipulation is performed.

We report herein the isolation and sequencing of the full-length mouse *Ceacam1* and *Ceacam2* genes. They both encode nine exons, with significant sequence differences in certain regions. These sequence differences will allow selective targeting of one *Ceacam* gene compared with another. In addition, the tissue-specific distributions of CEACAM1 and CEACAM2 were different, and only *Ceacam1* was expressed during embryonic development. The differences in their tissue and developmental expression patterns suggest that these two genes may have different functions *in vivo*.

## MATERIALS AND METHODS

### Isolation and characterization of *Ceacam1* and *Ceacam2*

A 129 Sv/Ev mouse genomic library in λLEX (provided by Dr Li-Yuan Yu-Lee, Baylor College of Medicine, Houston, TX,

Abbreviations used: CAM, cell-adhesion molecule; RT-PCR, reverse transcription–PCR.

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The *Ceacam1* and *Ceacam2* sequences have been deposited in the GenBank®/EMBL/DDBJ/GSDB Nucleotide Sequence Databases with accession numbers AF287911 and AF287912 respectively.

U.S.A.) was screened with the 1.6 kb full-length mouse *Ceacam1* cDNA [18], which had been labelled by using the Klenow fragment of DNA polymerase I, random hexanucleotide primers and [$\alpha$-$^{32}$P]dCTP [19]. A total of 24 positive clones were identified in the initial screening of this genomic library. PCR with Oligo 127 (5′-GTGTCACTCTAGGCTACAGGAAAT-3′) and Oligo 122 (5′-GAGGCCAGCTCCATGTCTCTGCTG-3′), which are specific to the 5′ region of mouse *Ceacam1* [20], showed that seven of the clones had the N-terminal *Ceacam1* sequence. Similarly, PCR with Oligo 120 (5′-GAAGTCTGGCGGATCT-GGCTCCTT-3′) and Oligo 131 (5′-TTGAAGTTCAGGACA-GTGTATGCG-3′) showed that seven clones had the 3′ *Ceacam1* sequence. These clones were isolated by secondary and tertiary screening and characterized by restriction mapping. The nucleotide sequences of the exons and introns were determined by primer walking using specific oligonucleotide primers. Sequencing was performed by the DNA Sequencing Core Facilities at M. D. Anderson Cancer Center with an automated fluorescent DNA sequencer (Applied Biosystems Inc., Ramsey, NJ, U.S.A.).

### Analysis of *Ceacam1* and *Ceacam2* expression using a mouse cDNA panel

A mouse cDNA panel containing first-strand cDNA prepared from mouse tissues and normalized for $\beta$-actin expression was purchased from OriGene Technologies Inc. (Rockville, MD, U.S.A.) and used to analyse the expression of *Ceacam1* and *Ceacam2* in various mouse tissues. Oligonucleotides specific to exon 2 of *Ceacam1* (Oligo 116, 5′-AATCTGCCCCTGGCG-CTTGGAGCC-3′; Oligo 179, 5′-AAATCGCACAGTCGCC-TGAGTACG-3′) and to exon 2 of *Ceacam2* (Oligo 117, 5′-AATATGATGAAGGGAGTCTTGGCC-3′; Oligo 180, 5′-AAATTGTCCAGTCAGGACCCTACG-3′) were used as primers to detect specific mRNAs for *Ceacam1* and *Ceacam2* by PCR. PCR cycling conditions were as follows: (1) pre-denaturation (94 °C, 3 min) for one cycle; (2) denaturation (94 °C, 30 s), annealing (60 °C, 30 s) and extension (72 °C, 2 min) for 35 cycles; and (3) final extension (72 °C, 5 min). The predicted size of the PCR products was 246 bp. These PCR products were analysed by agarose-gel electrophoresis, transferred on to a nylon membrane and hybridized with $^{32}$P-labelled oligonucleotide probes specific to *Ceacam1* (Oligo 181; 5′-AACAC-TACGGCTATAGACAAA-3′) and *Ceacam2* (Oligo 182; 5′-TCTACTACGTCTACAAATGCT-3′).

### *Ceacam2* cDNA sequence from mouse testis

RNA was prepared from mouse testis by using RNAzol B (TEL-TEST Inc. Friendswood, TX, U.S.A.). The cDNAs coding for *Ceacam2* were obtained from the testis RNA by reverse transcription–PCR (RT-PCR) with Oligo 630 (5′-GAATTCA-AGCTTAAGAAGCTAGCAGGCAGCAGAGAC-3′), which contains nucleotides −36 to +1 of exon 1, and Oligo 631 (5′-GCGGCCGCCTAATGATGATGATGATGATGATGCT-TCTTTTTTACTTCTGAATAAAC-3′), which is complementary to the end of the coding sequence in exon 9 plus seven histidine codons. RT-PCR was performed with Oligo 630 and Oligo 631 according to the procedures provided by the manufacturer (Amersham/Pharmacia Corp., Arlington Heights, IL, U.S.A.). The PCR product was subcloned into pCRII-topo (Invitrogen, San Diego, CA, U.S.A.) and its sequence was determined.

## RESULTS

### Isolation and characterization of mouse *Ceacam1* and *Ceacam2* genes

The seven clones with the 5′ sequence of *Ceacam1* had different restriction maps, as did another seven clones with the 3′ end. The different restriction maps seemed to reflect different genes. Grouping these clones according to their restriction maps and DNA sequences revealed two distinct sequences, representing *Ceacam1* and *Ceacam2*. The overlapping genomic clones that spanned *Ceacam1* and *Ceacam2* are shown in Figure 1. DNA sequence analysis of both strands of these two genes revealed that *Ceacam1* and *Ceacam2* each contain nine exons each, with sizes of approx. 18.3 and 24.65 kb respectively (Figure 1).

The sizes of the exons and introns and the intron/exon boundary sequences for *Ceacam1* and *Ceacam2* are shown in Tables 1 and 2 respectively. The first exon, which codes for the first two-thirds of the signal sequence, is 304 bp in both *Ceacam1* and *Ceacam2*. In contrast, exons 2–5 are each approx. 300 bp, and each codes for one Ig-like domain in both *Ceacam1* and *Ceacam2*. The transmembrane domain is encoded by exon 6 and the cytoplasmic domain by exons 7–9. Consistent with the RNA splicing rule [21], each intron starts with GT at the 5′ end and ends with AG at the 3′ end (Tables 1 and 2).

### Comparison of *Ceacam1* and *Ceacam2* genes

The overall similarity between *Ceacam1* and *Ceacam2*, including all exons and introns, is approx. 79.6 %. The similarities between

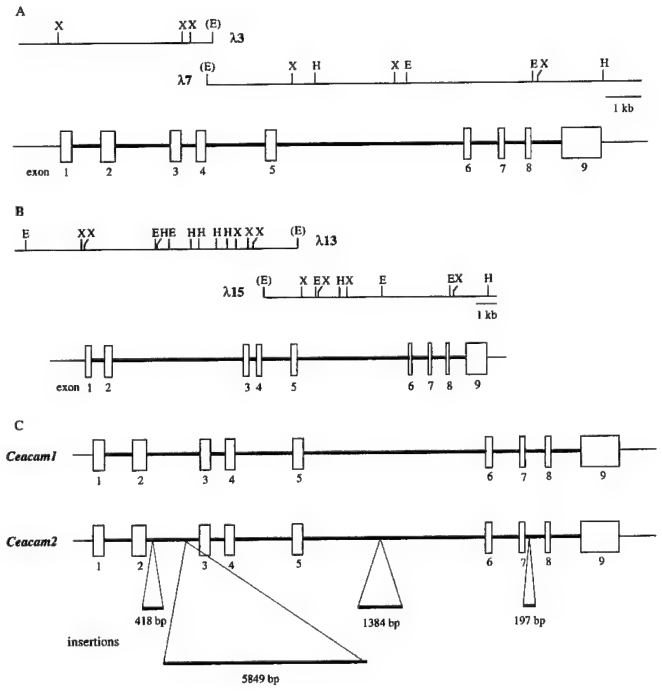



**Figure 1 Structures of the mouse *Ceacam1* and *Ceacam2* genes**

Numbered open boxes indicate exons. The thick lines connecting the exons represent introns. (**A**) The maps of two overlapping $\lambda$ clones ($\lambda$3 and $\lambda$7) that contain *Ceacam1*. (**B**) The maps of two overlapping $\lambda$ clones ($\lambda$13 and $\lambda$15) that contain *Ceacam2*. The restriction maps are aligned with the intron/exon map. E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. The enzyme sites in parentheses are from the multiple cloning region of the $\lambda$ phage vector. (**C**) Comparison of the mouse *Ceacam1* and *Ceacam2* genes.

**Table 1 Intron/exon boundaries of *Ceacam1***

| Exon | Size (bp) | Exon 3′ | | Intron | Size (bp) | | Exon 5′ | |
|---|---|---|---|---|---|---|---|---|
| 1 | 304 | ACA G<br>Thr A | gtaaggagatattcc ... | 1 | 767 | ... tctcttccctcttag | CC TCA<br>la Ser | |
| 2 | 360 | CAC C<br>His P | gtaagtaattatctg ... | 2 | 1540 | ... ctattatctgcacag | CC ATA<br>ro Ile | |
| 3 | 285 | ATC T<br>Ile T | gtgagtaacttcttc ... | 3 | 467 | ... ttctgtttgctccag | AT GGT<br>yr Gly | |
| 4 | 255 | CTT G<br>Leu G | gtaagtggatctctg ... | 4 | 1727 | ... tctgtttgtccacag | AG CCA<br>ln Pro | |
| 5 | 276 | ATA T<br>Ile P | gtgagtgacctcgcc ... | 5 | 5358 | ... ttctttccctgacag | TT GAC<br>he Asp | |
| 6 | 121 | GGC GG<br>Gly Gl | gtaggacagtctttc ... | 6 | 796 | ... ctcatatttatttag | G GGA<br>y Gly | |
| 7 | 53 | CAC A<br>His A | gtaagtaaagccaat ... | 7 | 587 | ... ttcttcctcccctag | AT CTG<br>sn Leu | |
| 8 | 32 | AAC AAG<br>Asn Lys | gtgagcactgccact ... | 8 | 948 | ... ctctcatcctttcag | GTG GAT<br>Val Asp | |
| 9 | 1180 | | | | | | | |

**Table 2 Intron/exon boundaries of *Ceacam2***

| Exon | Size (bp) | Exon 3′ | | Intron | Size (bp) | | Exon 5′ | |
|---|---|---|---|---|---|---|---|---|
| 1 | 304 | ACA G<br>Thr A | gtaaggagatattcc ... | 1 | 766 | ... tctcttccctcttag | CC TCA<br>la Ser | |
| 2 | 360 | CAC A<br>His T | gtaagtaattctctg ... | 2 | 7807 | ... acacagtgcagacag | CC CTA<br>hr Leu | |
| 3 | 285 | ATC T<br>Ile T | gtgagtaacttcttt ... | 3 | 465 | ... tcttgtttgctccag | AT GGT<br>yr Gly | |
| 4 | 255 | CTT G<br>Leu G | gtaagtggatctctg ... | 4 | 1717 | ... tctgtttgtccacag | AG CCA<br>lu Pro | |
| 5 | 276 | ATA T<br>Ile P | gtgagtgaccttgcc ... | 5 | 6737 | ... ttctttccctgacag | TT GAC<br>he Asp | |
| 6 | 118 | CGC TG<br>Arg Tr | gtaggacagtctttc ... | 6 | 798 | ... ctcatatttatttag | G GGA<br>p Gly | |
| 7 | 53 | CAC A<br>His A | gtaagtaaagccaat ... | 7 | 771 | ... ctcttcctcccctag | AT CTG<br>sn Leu | |
| 8 | 32 | AAC AAG<br>Asn Lys | gtgagcactgccact ... | 8 | 973 | ... ctctcatattttcag | GTG GAT<br>Val Asp | |
| 9 | 1180 | | | | | | | |

**Table 3 Intron/exon lengths and similarities of *Ceacam1* and *Ceacam2***

| | Length (bp) | | | | Length (bp) | | |
|---|---|---|---|---|---|---|---|
| Exon | *Ceacam1* | *Ceacam2* | Similarity (%) | Intron | *Ceacam1* | *Ceacam2* | Similarity (%) |
| 1 | 304 | 304* | 88.9 | 1 | 767 | 766 | 97.4 |
| 2 | 360 | 360 | 76.9 | 2 | 1540 | 7807 | 29.7 |
| 3 | 285 | 285 | 97.9 | 3 | 467 | 465 | 94.7 |
| 4 | 255 | 255 | 99.6 | 4 | 1726 | 1717 | 97.9 |
| 5 | 276 | 276 | 87.0 | 5 | 5358 | 6737 | 59.8 |
| 6 | 121 | 118 | 78.5 | 6 | 796 | 798 | 98.5 |
| 7 | 53 | 53 | 100 | 7 | 587 | 771 | 64.5 |
| 8 | 32 | 32 | 100 | 8 | 948 | 973 | 94.3 |
| 9 | 1180 | 1180† | 86.8 | | | | |

* Includes 240 bp of promoter and 5′ untranslated sequence.
† Includes 889 bp of 3′ untranslated sequence.

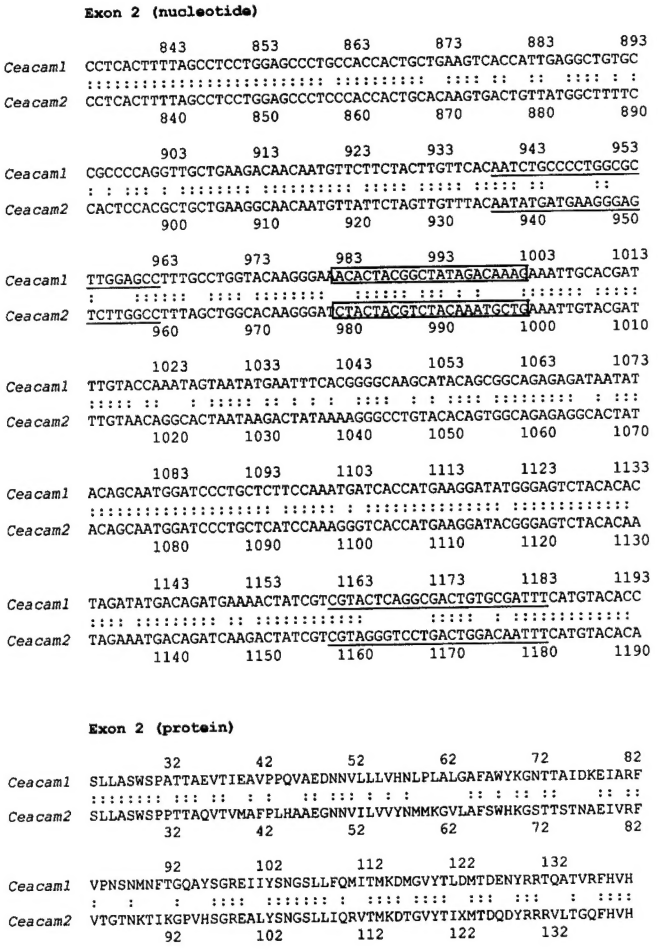



**Figure 2 Comparison of the nucleotide and protein sequences of exon 2 of *Ceacam1* and *Ceacam2***

Oligonucleotides for PCR analyses are underlined. Oligonucleotides for hybridization (Oligo 181 and Oligo 182) are boxed. The nucleotides are numbered with the A of the start ATG as the first nucleotide according to the genomic sequence. The amino acids are numbered with the start methionine (ATG) as the first amino acid.

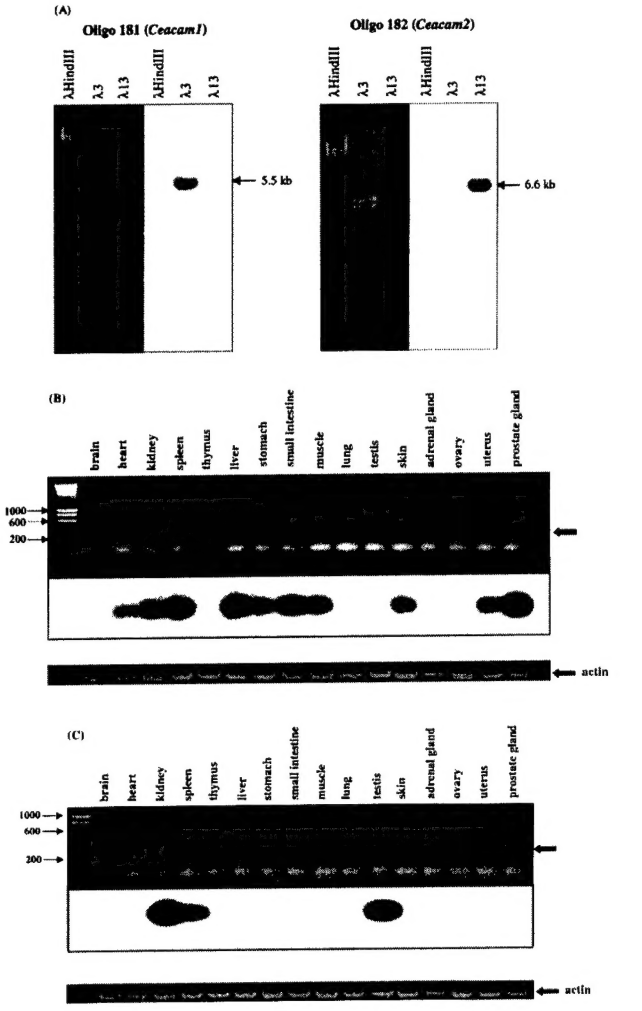


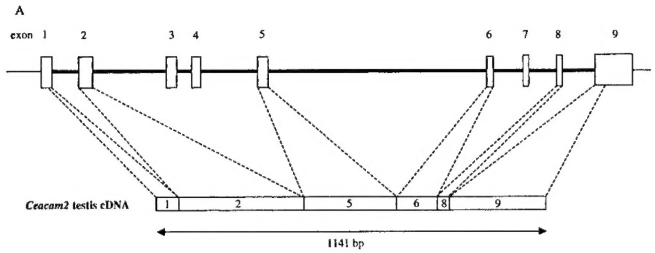

**Figure 3 Expression of *Ceacam1* and *Ceacam2* mRNAs in various mouse tissues**

(**A**) Specificity of Oligos 181 and 182 in distinguishing *Ceacam1* from *Ceacam2*. (**B**, **C**) Tissue-specific expression of *Ceacam1* (**B**) and *Ceacam2* (**C**) analysed by RT-PCR and hybridization with Oligo 181 and Oligo 182 respectively. The positions of the PCR products are indicated by the arrows on the right.

the exons and introns of *Ceacam1* and *Ceacam2* are shown in Table 3. The major differences between these two genes are in exon 2, intron 2, intron 5, exon 6 and intron 7, which are 76.9%, 29.7%, 59.8%, 78.5% and 64.5% similar respectively. The nucleotide and the deduced amino acid sequences of exon 2 for *Ceacam1* and *Ceacam2* are shown in Figure 2. It is apparent that most of the sequence substitutions in exons 2, which code for the first Ig domain, do result in amino acid substitutions in *Ceacam1* and *Ceacam2*. Thus the amino acid identity between the first Ig domains of CEACAM1 and CEACAM2 is 57.1%, which is significantly lower than the similarity of other domains. In addition, insertions of 418 bp and 5849 bp in intron 2, and of 1384 bp and 197 bp in introns 5 and 7 respectively, are found in *Ceacam2* compared with *Ceacam1* (Table 3 and Figure 1C).

## Expression of *Ceacam1* and *Ceacam2* mRNAs in mouse tissues

To determine the tissue-specific distribution of *Ceacam1* and *Ceacam2*, we performed semi-quantitative RT-PCR on a panel of mouse cDNAs from various tissues by using *Ceacam1*- and *Ceacam2*-specific oligonucleotide pairs. The PCR products were hybridized further with *Ceacam1*- and *Ceacam2*-specific oligonucleotides derived from exon 2, the least similar exon (Figure 2). The specificities of these two oligonucleotides were confirmed by Southern blot analysis. As shown in Figure 3(A), Oligo 181 hybridized to DNA from λ3 (*Ceacam1*) but not from λ13 (*Ceacam2*), whereas Oligo 182 only hybridized to λ13 DNA. Both *Ceacam1* and *Ceacam2* mRNAs were detected, although not in the same tissues. This observation supports the notion that both *Ceacam1* and *Ceacam2* are expressed in mouse (Figures 3B and 3C). High levels of *Ceacam1* mRNA were detected in liver, small intestine, prostate and spleen (Figure 3B), similar to the tissue expression pattern in humans [22] and rats [23,24]. *Ceacam1* mRNA was also detected in heart, kidney, stomach, muscle, skin and uterus. In contrast, *Ceacam2* mRNAs were detected only in kidney, testis and, to a lesser extent, spleen (Figure 3C). These




B

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-61 ATATCAGGGCAGCCAGGCTTAGCAGTAGTGTTGGAGAAGAAGCTAGCAGGCAGCAGAGAC  0

  1 ATGGAGCTGGCCTCAGCACATCTCCACAAAGGGCAGGTTCCCTGGTTTGGACTACTGCTC  60
    M  E  L  A  S  A  H  L  H  K  G  Q  V  P  W  F  G  L  L  L

 61 ACAGCCTCACTTTTAGCCTCCTGGAGCCCTCCCACCACTGCACAAGTGACTGTTATGGCT 120
    T  A  S  L  L  A  S  W  S  P  P  T  T  A  Q  V  T  V  M  A

121 TTTCCACTCCACGCCGCTGAAGGCAACAATGTTATTCTAGTTGTTTACAATATGATGAAG 180
    F  P  L  H  A  A  E  G  N  N  V  I  L  V  V  Y  N  M  M  K

181 GGAGTCTCGGCCTTTAGCTGGCACAAGGGATCTACTACGTCTACAAATGCTGAAATTGTA 240
    G  V  S  A  F  S  W  H  K  G  S  T  T  S  T  N  A  E  I  V

241 CGATTTGTAACAGGCACTAATAAGACTATAAAAGGGCCTGTACACAGTGGCAGAGAGACA 300
    R  F  V  T  G  T  N  K  T  I  K  G  P  V  H  S  G  R  E  T

301 CTATACAGCAATGGATCCCTGCTCATCCAAAGGGTCACCATGAAGGATACGGGAGTCTAC 360
    L  Y  S  N  G  S  L  L  I  Q  R  V  T  M  K  D  T  G  V  Y

361 ACAATAGAAATGACAGATCAAAGCTATCGTCGTAGGGTCCTGACTGGACAATTTCATGTA 420
    T  I  E  M  T  D  Q  S  Y  R  R  R  V  L  T  G  Q  F  H  V

421 CACAAGCCAGTGACTCAGCCCTCCCTCCAAGTCACCAACACCACAGTCAAAGAACTAGAC 480
    H  K  P  V  T  Q  P  S  L  Q  V  T  N  T  T  V  K  E  L  D

481 TCTGTCACCCTGACCTGCTTGTCGAAAGACCGTCAAGCCCACATCCATTGGATCTTCAAC 540
    S  V  T  L  T  C  L  S  K  D  R  Q  A  H  I  H  W  I  F  N

541 AATGATACTCTTCTAATCACAGAGAAGATGACAACCTCTCAGGCGGGACTCATCCTCAAA 600
    N  D  T  L  L  I  T  E  K  M  T  T  S  Q  A  G  L  I  L  K

601 ATAGACCCTATTAAGAGGGAAGATGCCGGCGAGTATCAGTGTGAAATCTCGAATCCAGTC 660
    I  D  P  I  K  R  E  D  A  G  E  Y  Q  C  E  I  S  N  P  V

661 AGCGTCAAGAGGAGCAACTCAATCAAACTGGAAGTAATATTTGACTCAACATATGACATT 720
    S  V  K  R  S  N  S  I  K  L  E  V  I  F  D  S  T  Y  D  I

721 TCAGATGTCCCCATTGCTGTAATCATAACTGGAGCTGTGGCCGGGGTGATTCTAATAGCA 780
    S  D  V  P  I  A  V  I  I  T  G  A  V  A  G  V  I  L  I  A

781 GGGCTGGCATATCGCCTCTGTTCCAGGAAGTCTCGCTGATCTGGCTCCTTCTGACAACTC 840
    G  L  A  Y  R  L  C  S  R  K  S  R  *

841 TCCTAACAAGGTGGATGACGTCGCATACACTGTCCTGAACTTCAATTCCCAGCAACCCAA 900

901 CCGGCCAACTTCAGCCCCTTCTTCTCCAAGAGCCACAGAAACAGTTTATTCAGAAGTAAA 960

961 AAAGAAG
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**Figure 4 *Ceacam2***

(**A**) cDNA structure. The numbered boxes indicate exons. The thick lines connecting the exons represent introns. The broken lines show the portions of the exon sequences included in testis *Ceacam2* cDNA. (**B**) cDNA and protein sequences.

observations demonstrate that the *Ceacam1* and *Ceacam2* genes are differentially expressed in mouse tissues.

### *Ceacam2* cDNA

Although it was detected in testis, kidney and spleen, *Ceacam2* mRNA may not contain open reading frames for protein translation. As testis expressed only *Ceacam2*, the cDNAs coding for *Ceacam2* were obtained from testis RNA by RT-PCR with Oligo 630, which contained the 5′ untranslated region of exon 1, and Oligo 631, complementary to the exon 9 coding region at the termination codon TGA. The 1 kb PCR product obtained only hybridized to *Ceacam2*-specific Oligo 182, and not to *Ceacam1*-specific Oligo 181, suggesting that the fragment codes for *Ceacam2*. This result is consistent with the tissue distribution of *Ceacam2* (Figure 3C). The PCR product was then subcloned

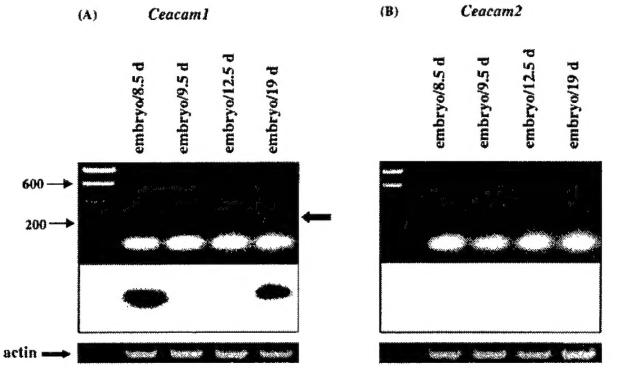


**Figure 5 Expression of *Ceacam1* (A) and *Ceacam2* (B) mRNAs during embryonic development**

(**A**) Analysis of RT-PCR products of *Ceacam1* on an agarose gel and gene-specific hybridization using Oligo 181. (**B**) Analysis of RT-PCR products of *Ceacam2* on an agarose gel and gene-specific hybridization using Oligo 182. Marker sizes are indicated by thin arrows, and the size of the PCR product is indicated by thick arrow.

and its sequence was determined (Figure 4). The mouse testis cDNA contained exons 1, 2, 5, 6, 8 and 9, and had an open reading frame of 273 amino acids (Figure 4B).

### Expression of *Ceacam1* and *Ceacam2* during mouse embryonic development

The expression of *Ceacam1* and *Ceacam2* was examined in mouse embryos at days 8.5, 9.5, 12.5 and 19 of embryonic development by RT-PCR hybridization as described above. Using *Ceacam1*-specific Oligo 181, we observed age-related differences in mRNA levels: we detected the mRNA at day 8.5, it had disappeared at days 9.5–12.5, and it re-appeared at day 19 (Figure 5A). In contrast, no hybridization signal was detected when *Ceacam2*-specific Oligo 182 was used (Figure 5B). This result suggests that the expression of *Ceacam1*, but not that of *Ceacam2*, is developmentally regulated.

## DISCUSSION

Because targeted gene deletion is performed in the 129 Sv mouse, we determined in the present studies the complete genomic structure and DNA sequences of the mouse *Ceacam1* and *Ceacam2* genes in a 129Sv/Ev mouse genomic library. We also examined the expression patterns of these two genes in adult mouse tissues and during embryonic development. Several conclusions can be made from this study. First, the *Ceacam1* and *Ceacam2* genes contain sufficient sequence differences that targeted gene deletion specific to either gene should be feasible. Secondly, functional redundancy may not be a problem when only one of these two genes is deleted, because, although *Ceacam1* and *Ceacam2* are highly homologous, they have different tissue expression patterns. Thirdly, *Ceacam1* is probably more important than *Ceacam2* in the mouse, because the tissue expression pattern of *Ceacam1* is similar to those of the single *Ceacam* genes in humans and rats. Conservation of the expression profile among these different species suggests that the function of

*Ceacam1* may be essential. Fourthly, *Ceacam2* plays no role in embryonic development; it is not expressed in the mouse embryo.

The results of the present study provide important information for the design of gene targeting strategies for functional studies of *Ceacam* genes, by either deleting or introducing mutations into the *Ceacam* genes in the mouse germline. The differences in the two DNA sequences can be used to target a specific gene. As *Ceacam1* lacks the 418 and 5849 bp insertions in *Ceacam2* intron 2 and the 1384 bp insertion present in *Ceacam2* intron 5 (Figure 1C), gene-targeting vectors containing introns 2 or 5 of *Ceacam1* or *Ceacam2* could be used to achieve selective homologous recombination in the desired gene. If mutant mice in which only one *Ceacam* gene is deleted survive, then they can be crossed further to generate mice deficient in both *Ceacam* genes. However, as *Ceacam1* and *Ceacam2* are both on chromosome 7, it is possible that they are too close to allow construction of double-gene-knockout mice by crossing the single-gene-knockout mice.

The existence of the highly similar second *Ceacam* gene, *Ceacam2*, in mice raises the possibility that *Ceacam2* is a pseudogene. However, our results indicate that *Ceacam2* is probably not a pseudogene, since (1) *Ceacam2* contains a complete set of exons and introns typical of a *Ceacam* gene; (2) *Ceacam2* is transcribed in mouse, as shown by the presence of *Ceacam2* mRNA in several tissues; and (3) *Ceacam2* mRNA contains an open reading frame of 273 amino acids in testis, as has been shown in CMT-93 mouse rectal carcinoma cells [17]. These findings raise the interesting question of the function of *Ceacam2*. One of the functions of CEACAM1 is inhibition of tumour growth [3,6]. This suggests that this protein may play an important role in regulating cell growth and differentiation. Structural and functional analyses of rat CEACAM1 have revealed that the tumour-suppressive function requires a long cytoplasmic domain, generated by alternative splicing [4,6,24]. Because the cytoplasmic domains of CEACAM1 and CEACAM2 are identical, CEACAM2 may also have growth-suppressive activity. However, the role of CEACAM2 in testis, which is composed of rapidly dividing cells in spermatogenesis, is not clear. Thus the *in vivo* function of CEACAM2 may not be revealed until its gene is deleted in the mouse.

Knocking out a gene may have no phenotypic effect if related genes have similar functions. We showed that *Ceacam1* and *Ceacam2* are expressed in different tissues in the mouse. *Ceacam1* mRNA was detected in tissues rich in epithelial cells, which is consistent with expression of the CEACAM1 homologue in rats and humans [25–27]. In contrast, *Ceacam2* mRNA was abundantly expressed in testis, which does not express *Ceacam1* mRNA. In addition, *Ceacam2* mRNA was undetectable in the mouse embryo, whereas *Ceacam1* mRNA was developmentally regulated. Thus it appears that the two mouse *Ceacam* genes are not functionally redundant. However, we cannot rule out the possibility that the loss of *Ceacam1* expression may up-regulate expression of *Ceacam2*. These studies will have to await targeted gene deletion of these two related genes.

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